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WITNESS my hand this
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PROVISIONAL SPECIFICATION

Invention Title: EMBRYONIC STEM CELLS

Applicant: MONASH UNIVERSITY, NATIONAL UNIVERSITY OF SINGAPORE & HADASIT MEDICAL RESEARCH SERVICES & DEVELOPMENT CO LTD

The invention is described in the following statement:

EMBRYONIC STEM CELLS

The present invention relates to undifferentiated embryonic stem cells, methods of cultivation and propagation, production of differentiated cells and uses thereof.

The promotion of stem cells capable of being maintained in an undifferentiated state *in vitro* allows for the study of the cellular and molecular biology of early human development, functional genomics, generation of differentiated cells from the stem cells for use in transplantation or drug screening and drug discovery *in vitro*.

In general, stem cells are undifferentiated cells which can give rise to a succession of mature functional cells. For example, a haematopoietic stem cell may give rise to any of the different types of terminally differentiated blood cells. Embryonic stem (ES) cells are derived from the embryo and are pluripotent, thus possessing the capability of developing into any organ, cell type or tissue type or, at least potentially, into a complete embryo. ES cells will differentiate into embryonic cells or somatic cells. However, the trigger that determines the cell type that results is unknown.

Mouse ES cells have successfully been maintained in an undifferentiated state using fibroblast feeder layers in cultivation in the presence of leukaemia inhibitory factor (LIF). If LIF is removed, mouse ES cells will differentiate, into embryonic and extra extra embryonic cells.

Mouse ES cells are indeed different to human ES cells. This is evident from the requirements necessary to maintain the cells in an undifferentiated state. Whilst fibroblast feeder layers or LIF are sufficient to prevent differentiation of mouse ES cells and allows continuous passage, this is insufficient for human ES cells. High concentrations of cloned LIF fail to prevent differentiation of primate ES cells in the absence of fibroblast feeder layer. Pluripotent human embryonal carcinoma cells and cells isolated from the human blastocyst similarly cannot be serially cultivated in the presence of LIF alone. Cultivation of undifferentiated human ES cells has previously failed to

produce more than a couple of passages before differentiation becomes evident.

It is an object of the invention to overcome or at least alleviate some of the problems of the prior art.

SUMMARY OF THE INVENTION

In one aspect of the present invention, there is provided a purified preparation of human embryonic stem cells capable of proliferation *in vitro* for an extended period.

In another aspect, there is provided a somatic cell differentiated *in vitro* from an undifferentiated embryonic stem cell.

Preferably the undifferentiated cells have the potential to differentiate when subjected to differentiating conditions.

More preferably, the undifferentiated cells are capable of maintaining an undifferentiated state when cultured on a fibroblast feeder layer.

In another aspect of the present invention there is provided an undifferentiated human embryonic stem cell wherein the cell is immunoreactive with markers for human pluripotent stem cells including SSEA-4, GCTM-2 antigen, TRA 1-60 and CD30. Preferably, the cells express the transcription factor Oct-4 as demonstrated by RT-PCR. More preferably, the cells maintain a diploid karyotype during prolonged cultivation *in vitro*.

In a further aspect of the present invention there is provided a method of preparing undifferentiated human embryonic stem cells, said method including:

obtaining *in vitro* fertilised human embryo;

removing inner cell mass (ICM) cells from the embryo;

culturing ICM cells on a fibroblast feeder layer to obtain stem cells; and

removing stem cells from the feeder layer.

In a preferred aspect of the invention the method further includes the following steps before removal of inner cell mass cells, said steps including:

treating the embryo to dislodge the trophectoderm of the embryo or a portion thereof;

washing the embryo with an appropriate blastocysts culture medium; for example G2 or S2 (Scandinavian-2 medium) to dislodge the trophectoderm or a portion thereof; and

obtaining inner cell mass cells of the embryo.

Preferably, the treatment of the embryo includes treating with an antibody or antiserum reactive with epitopes on the surface of the trophectoderm. More preferably, the treatment with antibody or antiserum is combined with treatment with complement. Most preferably, the combined antibody and complement are either anti-placental alkaline phosphatase antibody combined with Baby Rabbit complement; or antihuman serum antibody combined with Guinea Pig complement. The antibody and complement may be used together or separately to treat the embryo to dislodge the trophectoderm or a portion thereof.

In a further aspect of the invention, the method further includes:

replacing the stem cells from the fibroblast feeder layer onto another fibroblast feeder layer; and

culturing the stem cells for a period sufficient to obtain morphologically undifferentiated stem cells.

In an even further aspect of the invention the method further includes propagating the undifferentiated stem cells. The methods of propagation may initially involve dispersing stem cells from clumps representing colonies of cells. The dispersion is preferably by chemical or mechanical means. More preferably, the cells are treated chemically and washed in a PBS or they are mechanically severed from the colonies or a combination of the two methods.

In another aspect there is provided an undifferentiated cell line produced by the method of the present invention.

Preferably, the undifferentiated cell line is preserved by preservation methods such as cryopreservation. Preferably the method of cryopreservation includes the Open Pulled Straw (OPS) vitrification method.

In another aspect of the invention there is provided a method of induction of differentiation of stem cells. Preferably the method provides for induction of somatic cells from embryonic stem cells.

In a further aspect of the invention, there is provided a method of producing large quantities of differentiated and undifferentiated cells.

FIGURES

Figure 1 shows a colony of undifferentiated human ES cell line HES-1.

Figure 2 shows a colony from the same cell line which has undergone differentiation.

Figure 3 shows phase contrast micrographs of ES cells and their differentiated progeny. A, inner cell mass three days after plating. B, colony of ES cells. C, higher magnification of an area of an ES cell colony. D, an area of an ES cell colony undergoing spontaneous differentiation during routine passage. E, a colony four days after plating in the absence of a feeder cell layer but in the presence of 2000 units/ml human LIF undergoing differentiation in its periphery,. F, neuronal cells in a high density culture. Scale bars: A and C, 25 microns; B and E, 100 microns; D and F, 50 microns.

Figure 4 shows marker expression in ES cells and their differentiated progeny. A, ES cell colony showing histochemical staining for alkaline phosphatase. B. ES cell colony stained with antibody MC-813-70 recognising the SSEA-4 epitope. C, ES cell colony stained with antibody TRA1-60. D, ES cell colony stained with antibody GCTM-2. E, high density culture, cell body and processes of a cell stained with anti-neurofilament 68kDa protein. F, high density culture, cluster of cells and network of processes emanating from them stained with antibody against neural cell adhesion molecule. G, high density culture, cells showing cytoplasmic filaments stained with antibody to muscle actin. H, high density culture, cell showing cytoplasmic filaments stained with antibody to desmin. Scale bars: A, 100 microns; B-D, and F, 200 microns; E, G and H, 50 microns.

Figure 5 shows RT-PCR analysis of the expression of Oct-4 and beta-actin in ES stem cells and high density cultures. 1.5% agarose gel stained with ethidium bromide. Lane 1, DNA markers. Lane 2, stem cell culture, beta actin. Lane 3, stem cell culture, Oct-4. Lane 4, stem cell culture, PCR for Oct-4

carried out with omission of reverse transcriptase. Lane 5, high density culture, beta actin. Lane 6, high density culture, Oct-4. Lane 7, high density culture, PCR for Oct-4 carried out with omission of reverse transcriptase. Beta actin band is 200 bp and Oct-4 band is 320 bp.

Figure 6 shows histology of differentiated elements found in teratomas formed in the testis of SCID mice following inoculation of HES-1 or HES-2 colonies. A, cartilage and squamous epithelium, HES-2. B, neural rosettes, HES-2. C, ganglion, gland and striated muscle, HES-1. D, bone and cartilage, HES-1. E, glandular epithelium, HES-1. F, ciliated columnar epithelium, HES-1. Scale bars: A-E, 100 microns; F, 50 microns.

DETAILED DESCRIPTION OF THE INVENTION

In one aspect of the present invention there is provided a purified preparation of human undifferentiated embryonic stem cells capable of proliferation *in vitro* for an extended period of time.

The extended period may include several passages of cells. The cells are substantially maintained in an undifferentiated state.

Preferably the cells have the potential to differentiate when subjected to differentiating conditions.

More preferably, they are capable of maintaining an undifferentiated state when cultured on a fibroblast feeder layer preferably under non-differentiating conditions.

The promotion of stem cells capable of being maintained in an undifferentiated state *in vitro* allows for the study of the cellular and molecular biology of early human development, functional genomics, generation of differentiated cells from the stem cells for use in transplantation or drug screening and drug discovery *in vitro.* Until now, successful maintenance of the human stem cells in an undifferentiated state has not been achieved successfully.

Once the cells are maintained in the undifferentiated state, they may be differentiated to mature functional cells. The embryonic stem cells are derived

from the embryo and are pluripotent and have the capability of developing into any organ or tissue type. Preferably the tissue type is selected from the group including blood cells, neuron cells or muscle cells.

In another aspect of the present invention there is provided an undifferentiated human embryonic stem cell wherein the cell is immunoreactive with markers for human pluripotent stem cells including SSEA-4, GCTM-2 antigen, TRA 1-60 and CD30. Preferably, the cells express specific transcription factors such as Oct-4 as demonstrated by RT-PCR, or methods of analysis of differential gene expression, microarray analysis or related techniques. More preferably the cells maintain a diploid karyotype during prolonged cultivation *in vitro*.

Preferably, the stem cell will constitute a purified preparation of an undifferentiated stem cell line. More preferably, the stem cell line is a permanent cell line, distinguished by the characteristics identified above. They preferably have normal karyotype along with the characteristics identified above. This combination of defining properties will identify the cell lines of the invention regardless of the method used for their isolation.

Methods of identifying these characteristics may be by any method known to the skilled addressee. Methods such as (but not limited to) indirect immunoflourescence or immunocytochemical staining may be carried out on colonies of ES cells which are fixed by conventional fixation protocols then stained using antibodies against stem cell specific antibodies and visualised using secondary antibodies conjugated to fluorescent dyes or enzymes which can produce insoluble colored products. Alternatively, RNA may be isolated from the stem cells and PR-PCR or Northern blot analysis carried out to determine expression of stem cell specific genes such as Oct-4.

In a preferred embodiment the undifferentiated cells form tumours when injected in the testis of immunodeprived SCID mice; these tumours include differentiated cells representative of all three germ layers. The germ layers are preferably endoderm, mesoderm and ectoderm. Preferably, once the tumours are established, they may be disassociated and specific differentiated cell types may be identified or selected by any methods available to the skilled

addressee. For instance, lineage specific markers may be used through the use of fluorescent activated cell sorting (FACS) or other sorting method or by direct micro dissection of tissues of interest. These differentiated cells may be used in any manner. They may be cultivated *in vitro* to produce large numbers of differentiated cells which could be used for transplantation or for use in drug screening for example.

In another preferred embodiment, the undifferentiated cells differentiate *in vitro* to form somatic cells.

The cells may undergo differentiation *in vitro* to yield somatic cells as well as extrembryonic cells, such differentiation being characterised by novel gene expression characteristic of specific lineages as demonstrated by immunocytochemical or RNA analysis. Characterisation may be obtained by using expression of genes characteristic of pluripotent cells or particular lineages. Preferably, differential expression of Oct-4 and Pax-6 or nestin may be used to identify stem cells from differentiated cells. Otherwise, the presence or absence of expression of other genes characteristic of pluripotent stem cells or other lineages may include Genesis, GDF-3 or Cripto. Analysis of these gene expressions may create a gene expression profile. Methods of analysis include RT-PCR, methods of differential gene expression, microarray analysis or related techniques.

Differentiating cultures of the stem cells secrete HCG and AFP into culture medium, as determined by enzyme-linked immunosorbent assay carried out on culture supernatants. Hence this may also serve as a means of identifying the differentiated cells.

The differentiated cells forming somatic cells may also be characterised by expressed markers characteristic of differentiating cells. The *in vitro* differentiated cell culture may be differentiated into a single somatic cell type or it may differentiate into multiple somatic lineages. These multiple lineages may also be identified by detecting molecules selected from the group including neural cell adhesion molecule, neuro-filament proteins, desmin and smooth muscle action. In a further aspect of the present invention there is

provided a method of preparing undifferentiated human embryonic stem cells, said method including:

obtaining an *in vitro* fertilised human embryo;

removing inner cells mass (ICM) cells from the embryo;

culturing ICM cells on a fibroblast feeder layer to obtain stem cells; and

removing stem cells from the feeder layer.

Embryonic stem cells (ES) are derived from the embryo. These cells are undifferentiated and have the capability of differentiation to a variety of cell types. The "embryo" is defined as any stage after fertilization up to 8 weeks post conception. It develops from repeated division of cells and includes the stages of a blastocyst stage which comprises an outer trophectoderm and an inner cell mass (ICM).

The embryo required in the present method is an *in vitro* fertilised embryo.

The embryo may be fertilised by any *in vitro* methods available. For instance, the embryo may be fertilised by using conventional insemination, or intracytoplasmic sperm injection. It is preferred that any embryo culture method is employed but it is most preferred that a method producing high quality (good morphological grade) of blastocysts is employed. The high quality of the embryo can be assessed by morphological criteria. Most preferably the inner cell mass is well developed. These criteria can be assessed by the skilled addressee.

Following insemination, embryos may be cultured to the blastocyst stage. This stage may be assessed to determine suitable embryos for deriving ICM cells. The embryos may be cultured in any medium that maintains their survival and enhances blastocyst development.

Preferably, the embryos are cultured in droplets under pre-equilibrated sterile mineral oil in IVF-50 or S1 or G1.2 medium (Scandinavian IVF). Preferably the incubation is for two days. On approximately the third day, an appropriate medium may be used such as a mixture of 1:1 of IVF-50 and Scandinavian-2 medium (Scandinavian IVF) may be used. From at least the

fourth day, a suitable medium such as G2.2 or S2 Scandinavian-2 medium may be used solely to grow the embryos to blastocyst stage (blastocysts).

In a preferred embodiment, the blastocyst is subjected to enzymatic digestion to remove the zona pellucida or a portion thereof. Preferably the blastocyst is subjected to the digestion at an expanded blastocyst stage. Generally this is at approximately six days after insemination.

Any protein enzyme may be used to digest the zona pellucida or portion thereof from the blastocyst. Examples include pronase, acid Tyrodes solution, and mechanical methods such as laser dissection.

Preferably, Pronase is used. The pronase may be dissolved in PBS and G2 or S2 medium Scandinavian-2 medium. Preferably the PBS and Scandinavian-2 medium is diluted 1:1. For digestion of zone pellucida from the blastocyst, approximately 10 units/ml of Pronase may be used for a period sufficient to remove the zona pellucida. Preferably approximately 1 to 2 mins, more preferably 1-1.5 mins is used.

The embryo (expanded blastocyst) may be washed in G2.2 or S2 medium (Scandinavian-2) medium, and further incubated to dissolve the zona pellucida. Preferably, further digestion steps may be used to completely dissolve the zona. Removal of the zona pellucida thereby exposes the ICM (and trophectoderm (TE)).

In a preferred aspect of the invention the method further includes the following steps before removal of inner cell mass cell, said steps including:

treating the embryo to dislodge the trophectoderm of the embryo or a portion thereof;

washing the embryo with a G2.2 or S2 (Scandinavian-2) medium to dislodge the trophectoderm or a portion thereof; and

obtaining inner cell mass cells of the embryo.

Having had removed the zona pellucida, the ICM and trophectoderm become accessible. Preferably the trophectoderm is separated from the ICM. Any method may be employed to separate the trophectoderm from the ICM. Preferably the embryo (or blastocyst devoid of zona pellucida) is subjected to immuno-surgery. Preferably it is treated with an antibody or antiserum reactive

with epitopes on the surface of the trophectoderm. More preferably, the treatment of the embryo, (preferably an embryo at the blastocyst stage devoid of zona pellucida) is combined with treatment with complement. The antibody and/or antiserum and complement treatment may be used separately or together. Preferred combinations of antibody and/or antiserum and complement include anti-placental alkaline phosphatase antibody and Baby Rabbit complement (Serotec) or anti-human serum antibody (Sigma) combined with Guinea Pig complement (Gibco).

Preferably the antibodies and complement are diluted in G2.2 or S2 (Scandinavian-2) medium. The antibodies and complement, excluding anti-placental alkaline phosphate (anti-AP) are diluted 1:5 whereas anti-AP antibody is diluted 1:20 with Scandinavian-2 medium.

Preferably the embryo or blastocyst (preferably having the zona pellucida removed) is subjected to the antibody before it is subjected to the complement. Preferably, the embryo or blastocyst is cultured in the antibody for a period of approximately 30 mins.

Following the antibody exposure, it is preferred that the embryo is washed. Preferably it is washed in G2.2 or S2 (Scandinavian-2) medium. The embryo or blastocyst preferably is then subjected to complement, preferably for a period of approximately 30 mins.

G2.2 or S2 (Scandinavian-2) medium is preferably used to wash the embryo or blastocyst to dislodge the trophectoderm or a portion thereof. Dislodgment may be by mechanical means. Preferably the dislodgment is by pipetting the blastocyst through a small bore pipette.

The ICM cells may then be exposed and ready for removal and culturing. Culturing of the ICM cells is conducted on a fibroblast feeder layer. In the absence of a fibroblast feeder layer, the cells will differentiate. Leukaemia inhibitory factor (LIF) has been shown to replace the feeder layer in some cases and maintain the cells in an undifferentiated state. However, this seems to only work for mouse cells. For human cells, high concentration of LIF were unable to maintain the cells in an undifferentiated state in the absence of a fibroblast feeder layer.

Mouse or human fibroblasts are preferably used. They may be used separately or in combination. Human fibroblasts provide support for stem cells, but they create a non-even and sometimes non-stable feeder layer. Human fibroblasts are mostly preferred. However, they may combine effectively with mouse fibroblasts to obtain an optimal stem cell growth and inhibition of differentiation.

The cell density of the fibroblast layer affects its stability and performance. A combination of mouse and human fibroblasts is preferred. A density of approximately 25,000 human and 70,000 mouse cells per cm^2 is most preferred. The fibroblasts may be plated up to 48 hours before culturing the stem cells. Mouse fibroblasts alone are used at 75,000-100,000/cm^2. The feeder layers are preferably established 6-48 hours prior to addition of ES cells.

Preferably the mouse or human fibroblast cells are low passage number cells. The quality of the fibroblast cells affects their ability to support the stem cells. Embryonic fibroblasts are preferred. For mouse cells, they may be obtained from 13.5 day old foetuses. However, any source is suitable. Human fibroblasts may be derived from embryonic or foetal tissue from termination of pregnancy and may be cultivated using standard protocols of cell culture.

It is preferred that the cells are treated to arrest their growth. Several methods are available. It is preferred that they are irradiated or are treated with chemicals such as mitomycin C which arrests their growth. Most preferably, the fibroblast feeder cells are treated with mitomycin C.

The fibroblast feeder layer maybe generally plated on a gelatin treated dish. Preferably, the tissue culture dish is treated with 0.1% gelatin.

The fibroblast feeder layer may also contain modified fibroblasts. For instance, fibroblasts expressing recombinant membrane bound factors essential for stem cell renewal may be used. Such factors maybe CD30 ligand or Jagged 1 or human multipotent stem cell factor.

Inner cell mass cells may be cultured on the fibroblast feeder layer and maintained in an ES medium. A suitable medium is DMEM (GIBCO, without sodium pyruvate, with glucose 4500mg/L) supplemented with 20% FBS (Hyclone, Utah), (betamercaptoethanol - 0.1mM (GIBCO), non essential amino

acids - NEAA 1% (GIBCO), glutamine 2mM. (GIBCO), and penicillin 50µ/ml, streptomycin 50µg/ml (GIBCO). In the early stages of ES cell cultivation, the medium maybe supplemented with human recombinant leukemia inhibitory factor hLIF preferably at 2000µ/ml. However, LIF generally is not necessary. Any medium may be used that can support the ES cells.

The ES medium may be further supplemented with soluble growth factors which promote stem cell growth or survival or inhibit stem cell differentiation. Examples of such factors include human multipotent stem cell factor, or GM-CSF, or embryonic stem cell renewal factor.

The isolated ICM may be cultured for at least six days. At this stage, clumps of cells or colonies of cells develop. These colonies are clumps of principally undifferentiated stem cells. They may exist on top of differentiated cells. Isolation of the undifferentiated cells may be achieved by chemical or mechanical means of both. They may be removed mechanically by a micropipette or they maybe dispensed in a Ca^{2+}/Mg^{2+} free PBS medium or both.

In a further aspect of the invention, the method further includes:

replating the stem cells from the fibroblast feeder layer onto another fibroblast feeder layer; and

culturing the stem cells for a period sufficient to obtain morphologically undifferentiated stem cells.

A further replating of the undifferentiated stem cells is preferred. The isolated clumps of cells from the first fibroblast feeder layer may be replated on fresh human/mouse fibroblast feeder layer as described above.

Preferably, the cells are cultured for a period of 10-14 days. After this period, colonies of undifferentiated stem cells may be observed. The stem cells may be morphologically identified preferably by the high nuclear/cytoplasmic ratios, prominent nucleoli and compact colony formation. The cell borders are often distinct and the colonies are often flatter than mouse ES cells. The colonies resemble those formed by pluripotent human embryonal carcinoma cell lines such as GCT 27 X-1.

Other means of identifying the stem cells may be by cell markers or by measuring expression of genes characteristic of pluripoent cells.

Examples of genes characteristic of pluripotent cells or particular lineages may include (but are not limited to) Oct-4 and Pax-6 or nestin as markers of stem cells and neuronal precursors respectively. Other genes characteristic of stem cells may include Genesis, GDF-3 and Cripto. Such gene expression profiles may be attained by any method including RT-PCR, methods of differential gene expression, microarray analysis or related techniques.

Preferably the stem cells may be identified by being immunoreactive with markers for human pluripotent stem cells including SSEA-4, GCTM-2 antigen, TRA 1-60 or CD30. Preferably the cells express the transcription factor Oct-4. The cells may also maintain a diploid karyotype.

In yet another aspect of the present invention, the method further includes:

replating the stem cells in culture conditions that induce differentiation.

The stem cells are initially in an undifferentiated state and can be induced to differentiate. Generally the presence of a fibroblast feeder layer will maintain these cells in an undifferentiated state. This has been found to be the case with the cultivation of mouse and human ES cells. However, without being restricted by theory, it has now become evident that the type and handling of the fibroblast feeder layer is important for maintaining the cells in an undifferentiated state or inducing differentiation of the stem cells.

Somatic differentiation *in vitro* of the ES cell lines is a function of the period of cultivation following subculture, the density of the culture, and the fibroblast feeder cell layer. It has been found that somatic differentiation is morphologically apparent and demonstrable by immunochemistry approximately 14 days following routine subcultivation as described above in areas of the colony which are remote from direct contact with the feeder cell layer (in contrast to areas adjacent to the feeer cell layer where rapid stem cell growth is occuring such as the periphery of a colony at earlier time points after subcultivation), or in cultures which have reached confluence. Depending upon

the method of preparation and handling of the mouse embryo fibroblasts, the mouse strain from which the fibroblasts are derived, and the quality of a particular batch, stem cell renewal, extraembryonic differentiation or somatic differentiation may be favoured. Each batch of fibroblasts is routinely tested to determine its suitability for support of stem cell renewal, extraembryonic differentiation, or somatic differentiation.

Once a suitable fibroblast cell line is selected, it may be used as the differentiation inducing fibroblast feeder layer which induces the undifferentiated stem cells to differentiate into a somaticlineage or multiple somatic lineages. These may be identified using markers or gene expression as described above.

In an even further aspect of the invention, the method further includes propagating the undifferentiated stem cells. The methods of propagation may initially involve dispersing stem cells from clumps representing colonies of cells. The dispersion is preferably by chemical or mechanical means. More preferably, the cells are washed in a Ca^{2+}/Mg^{2+} free PBS or they are mechanically severed from the colonies or a combination of the two methods. In both methods, cells may be propagated as clumps of about 100 cells every 5-7 days.

In the first method, Ca^{2+}/Mg^{2+} free PBS medium may be used to reduce cell-cell attachments. Following about 15-20 minutes, cells gradually start to dissociate from the monolayer and from each other and desired size clumps can be isolated. When cell dissociation is partial, mechanical dissociation using the sharp edge of the pipette may assist with cutting and the isolation of the clumps.

An alternative chemical method may include the use of an enzyme. The enzyme may be used alone or in combination with a mechanical method. Preferably, the enzyme is dispase.

An alternative approach includes the combined use of mechanical cutting of the colonies followed by isolation of the subcolonies by dispase. Cutting of the colonies may be performed in PBS containing Ca^{2+} and Mg^{2+}. The sharp edge of a micropipette may be used to cut the colonies to clumps of

about 75-100 cells. The pipette may be used to scrape and remove areas of the colonies. The PBS is preferably changed to regular equilibrated human stem cell medium containing dispase (Gibco) 10 mg/ml and incubated for approximately 5minutes at 37°C in a humidified atmosphere containing 5% CO_2. As soon as the clumps detached they may be picked up by a wide bore micro-pipette, washed in PBS containing Ca^{2+} and Mg^{2+} and transferred to a fresh feeder layer.

The feeder layer may be as described above.

The stem cells may be further modified at any stage of isolation. They may be genetically modified through introduction of vectors expressing a selectable marker under the control of a stem cell specific promoter such as Oct-4 or CD30. These cells may be selected against differentiated cells. The presence of such cells may further induce stem cell differentiation.

The stem cells may be genetically modified at any stage with markers so that the markers are carried through to any stage of cultivation. The markers may be used to purify the differentiated or undifferentiated stem cell population at any stage of cultivation.

Progress of the stem cells and their maintenance in a differentiated or undifferentiated stage may be monitored in a quantitative fashion by the measurement of stem cell specific secreted products into the culture medium or in fixed preparations of the cells using ELISA or related techniques. Such stem cell specific products might include the soluble form of the CD30 antigen or the GCTM-2 antigen or they may be monitored as described above using cell markers or gene expression.

In another aspect of the invention there is provided a method of induction of differentiation of stem cells.

Undifferentiated ES cells in the right conditions will differentiate into the embryonic germ layers (endoderm, mesoderm and ectoderm). However, this differentiation process can be controlled. Preferably the timing of the cell type can be controlled.

The undifferentiated cell lines of the present invention may be cultured indefinitely until a differentiating signal is given.

Preferably, a differentiating signal inducing differentiation includes cultivating to high density or any condition unfavourable for continuous stem cell growth such as cultivation to high density or in the absence of a feeder cell layer. Once achieving confluence (ie. continuously covering the culture surface) the cells may spontaneously differentiate.

Differentiation may also be induced by cultivating to a high density in monolayer or on semi-permeable membranes so as to create structures mimicing the postimplantation phase of human development, or any modification of this approach. Cultivation in the presence of cell types representative of those known to modulate growth and differentiation in the vertebrate embryo (eg. endoderm cells or cells derived from normal embyronic or neoplastic tissue) may also induce differentiation or modulate differentiation so as to favour the establishment of particular cell lineages.

Chemical differentiation may also be used to induce differentiation. Propagation in the presence of soluble or membrane bound factors known to modulate differentiation of vertebrate embryonic cells, such as bone morphogenetic protein-2 or antagonists of such factors, may be used.

Applicants have found that Oct-4 is expressed in stem cells and down-regulated during differentiation and this strongly indicates that stem cell selection using drug resistance genes driven by the Oct-4 promoter will be a useful avenue for manipulating human ES cells. Directed differentiation using growth factors, or the complementary strategy of lineage selection coupled with growth factor enhancement could enable the selection of populations of pure committed progenitor cells from spontaneously differentiating cells generated as described here.

Genetic modification of the stem cells or further modification of those genetically modified stem cells described above may be employed to control the induction of differentiation. Genetic modification of the stem cells so as to introduce a construct containing a selectable marker under the control of a promoter expressed only in specific cell lineages, followed by treatment of the cells as described above and the subsequent selection for cells in which that promoter is active may be used.

In another aspect there is provided an undifferentiated cell line produced by the method of the present invention.

Specific cell lines HES-1 and HES-2 were isolated by the procedures described above and have the properties described above.

In another aspect of the invention, there is provided a differentiated cell produced by the methods of the present invention.

Once the cells have been induced to differentiate, the various cell types, identified by means described above, may be separated and selectively cultivated.

Selective cultivation means isolation of specific lineages of differentiated cells from mixed populations preferably appearing under conditions unfavourable for stem cell growth and subsequent propagation of these specific lineages. Isolation may be achieved by various techniques in cell biology including the following alone or in combination: microdissection; immunological selection by labelling with antibodies against epitopes expressed by specific lineages of differentiated cells followed by direct isolation under flourescence microscopy, panning, immunomagnetic selection, or selection by flow cytometry; selective conditions favouring the growth or adhesion of specific cell lineages such as exposure to particular growth or extracellular matrix factors or selective cell-cell adhesion; separation on the basis of biophysical properties of the cells such as density; disaggregation of mixed populations of cells followed by isolation and cultivation of small clumps of cells or single cells in separate culture vessels and selection on the basis of morphology, secretion of marker proteins, antigen expression, growth properties, or gene expression; lineage selection using lineage specific promoter constructs driving selectable markers or other reporters.

In another aspect of the invention there is provided a cell composition including a human differentiated or undifferentiated cell preferably produced by the method of the present invention, and a carrier.

The carrier may be any physiologically acceptable carrier that maintains the cells. It may be PBS or ES medium.

The differentiated or undifferentiated cells may be preserved or maintained by any methods suitable for storage of biological material. Vitrification of the biological material is the preferred method over the traditional slow-rate freezing methods. However, these traditional methods may also be employed to preserve the differentiated or undifferentiated cells. Effective preservation allows for continued storage of cells for future cultivation.

The Open Pulled Straw (OPS) vitrification method previously described by Vajta, G. et al (1998) Molecular Reproduction and Development, 51, 53-58, is preferred for cryopreserving the undifferentiated cells. More preferably, the method described by Vajta, G. et al (1998) Cryo-Letters, 19, 389 - 392 is employed. Generally, this method has only been used for cryopreserving embryos and it has been surprisingly found that the method, previously used for embryos is suitable for these cells. Instead of embryos, applicants have utilised clumps of undifferentiated cells to freeze using the methods identified above for embryos.

The differentiated or undifferentiated cells may be used as a source for isolation or identification of novel gene products, or for the generation of antibodies against novel epitopes. The cell lines may be used for the development of means to diagnose, prevent or treat congenital diseases.

Much attention recently has been devoted to the potential applications of stem cells in biology and medicine. The properties of pluripotentiality and immortality are unique to ES cells and enable investigators to approach many issues in human biology and medicine for the first time. ES cells potentially can address the shortage of donor tissue for use in transplantation procedures, particularly where no alternative culture system can support growth of the required committed stem cell. However, it must be noted that almost all of the wide ranging potential applications of ES cell technology in human medicine-based embryological research, functional genomics, growth factor and drug discovery, toxicology, and cell transplantation-are based on the assumption that it will be possible to grow ES cells on a large scale, to introduce genetic modifications into them, and to direct their differentiation. Present systems fall

short of these goals. The identification of novel factors driving pluripotential stem cell growth or stem cell selection protocols to eliminate the inhibitory influence of differentiated cells, both offer a way forward for expansion and cloning of human ES cells.

The present invention will now be more fully described with reference to the following examples. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

EXPERIMENTAL PROTOCOLS

1. Derivation and propagation of ES cells.

Fertilised oocytes were cultured to the blastocyst stage (day 6 after insemination), in sequential media, according to a standard co-culture free protocol (Fong C.Y., and Bongso A. Comparison of human blastulation rates and total cell number in sequential culture media with and without co-culture. *Hum. Reprod.* 14, 774-781 (1999)). After zona pellucida digestion by pronase (Sigma, St. Louis,MO)(Fong C.Y. et al. Ongoing pregnancy after transfer of zona-free blastocysts: implications for embryo transfer in the human. *Hum. Reprod.* 12, 557-560 (1997)), ICM were isolated by immunosurgery (Solter D., and Knowles, B. Immunosurgery of mouse blastocyst. *Proc. Natl. Acad. Sci. U.S.A.* 72, 5099-5102 (1975)) using anti-human serum antibody (Sigma) followed by exposure to guinea pig complement (Life Technologies, Gaithersburg, MD). ICM were then cultured on mitomycin C mitotically inactivated mouse embryonic fibroblast feeder layer (75,000 cells/cm2) in gelatine coated tissue culture dishes. The culture medium consisted of DMEM (Gibco, without sodium pyruvate, glucose 4500mg/L) supplemented with 20% fetal bovine serum (Hyclone, Logan, Utah), 0.1mM beta-mercaptoethanol, 1% non essential amino acids, 2mM glutamine, 50u/ml penicillin and 50μg/ml streptomycin (Life Technologies). During the isolation and early stages of ES cell cultivation, the medium was supplemented with human recombinant leukemia inhibitory factor hLIF at 2000u/ml (Amrad, Melbourne, Australia). 6-8 days after initial plating, ICM like clumps were removed mechanically by a

micropipette from differentiated cell outgrowths and replated on fresh feeder layer. The resulting colonies were further propagated in clumps of about 100 stem cell like cells, on mouse feeder layer, about every 7 days. The clumps were either dissociated mechanically, or with a combined approach of mechanical slicing followed by exposure to dispase (10mg/ml, Life Technologies).

2. Stem cell characterisation.

Colonies were fixed in the culture dishes by 100% ethanol for immuno-fluorescence demonstration of the stem cell surface markers GCTM-2, TRA 1-60 and SSEA-1, while 90% acetone fixation was used for SSEA-4. The sources of the monoclonal antibodies used for the detection of the markers were as follows: GCTM-2, this laboratory; TRA 1-60, a gift of Peter Andrews, University of Sheffield; SSEA-1 (MC-480) and SSEA-4 (MC-813-70), Developmental Studies Hybridoma Bank, Iowa, IA. Antibody localisation was performed by using rabbit anti-mouse immunoglobulins conjugated to fluorescein isothiocyanate (Dako,Carpinteria, CA).

Alkaline phosphatase activity was demonstrated as previously described (Buehr M. and Mclaren A. Isolation and culture of primordial germ cells. *Methods Enzymol.* 225, 58-76, (1993)). Standard G-banding techniques were used for karyotyping.

3. Oct-4 expression studies.

To monitor expression of Oct-4, RT-PCR was carried out on colonies consisting predominantly of stem cells, or colonies which had undergone spontaneous differentiation as described below. mRNA was isolated on magnetic beads (Dynal AS, Oslo) following cell lysis according to the manufacturer's instructions, and solid-phase first strand cDNA synthesis was performed using Superscript II reverse transcriptase (Life Technologies). As a control for mRNA quality, beta-actin transcripts were assayed using the same RT-PCR and the following primers: 5'-CGCACCACTGGCATTGTCAT-3'

(forward), 5'-TTCTCCTTGATGTCACGCAC-3' (reverse). Products were analysed on a 1.5% agarose gel and visualised by ethidium bromide staining.

4. In-vitro differentiation.

Colonies were cultured on mitotically inactivated mouse embryonic fibroblasts to confluency (about 2 weeks) and further on up to **7** weeks after passage. The medium was replaced every day. Alphafetoprotein and beta human chorionic gonadotropin levels were measured in medium conditioned by HES-1 and HES-2 at passage level 17 and 6 respectively. After 4-5 weeks of culture, conditioned medium was harvested 36 hours after last medium change, and the protein levels were determined by a specific immunoenzymometric assays (Eurogenetics, Tessenderllo, Belgium) and a fluorometric enzyme immunoassay (Dade, Miami, FL) respectively. These compounds were not detected in control medium conditioned only by feeder layer.

Differentiated cultures were fixed 6-7 weeks after passage (26 – HES-1 and 9 – HES-2) for immunofluorescence detection of lineage specific markers. After fixation with 100% ethanol, specific monoclonal antibodies were used to detect the 68 kDa neurofilament protein (Amersham, Amersham U.K), and neural cell adhesion molecule (Dako). Muscle specific actin and desmin were also detected by monoclonal antibodies (Dako) after fixation with methanol/acetone (1:1). Antibody localisation was performed as described above.

5. Teratoma formation in Severe Combined Immunodeficient (SCID) mice.

At the time of routine passage, clumps of about 200 cells with an undifferentiated morphology were harvested as described above, and injected into the testis of 4-8 week old SCID mice (CB17 strain from the Walter and Eliza Hall Institute, Melbourne, Australia, 10-15 clumps/testis). 6-7 weeks later, the resulting tumours were fixed in neutral buffered formalin 10%, embedded in paraffin and examined histologically after hematoxylin and eosin staining.

Example 1 - Derivation of cell lin s HES-1 and HES-2

The outer trophectoderm layer was removed from four blastocysts by immunosurgery to isolate inner cell masses (ICM), which were then plated onto a feeder layer of mouse embryo fibroblasts (Figure 3A). Within several days, groups of small, tightly packed cells had begun to proliferate from two of the four ICM. The small cells were mechanically dissociated from outgrowths of differentiated cells, and following replating they gave rise to flat colonies of cells with the morphological appearance of human EC or primate ES cells (Figure 3B, C stem cell colonies). These colonies were further propagated by mechanically disaggregation to clumps which were replated onto fresh feeder cell layers. Growth from small clumps of cells (<10 cells) was not possible under the conditions of these cultures. Spontaneous differentiation, often yielding cells with the morphological appearance of early endoderm, was frequently observed during routine passage of the cells (Figure 3D). Differentiation occurred rapidly if the cells were deprived of a feeder layer, even in the presence of LIF (Figure 3E). While LIF was used during the early phases of the establishment of the cell lines, it was subsequently found to have no effect on the growth or differentiation of established cultures (not shown). Cell line HES-1 has been grown for 35 passages in vitro and HES-2 for 15 passages, corresponding to a minimum of approximately 190 and 90 population doublings respectively, based on the average increase in colony size during routine passage, and both cell lines still consist mainly of cells with the morphology of ES cells. Both cell lines have been successfully recovered from cryopreservation.

Example 2 - Marker expression and karyotype of the human ES cells

Marker and karyotype analysis were performed on HES-1 at passage levels 5-7, 14-18 and 24-26, and on HES-2 at passage levels 6-8. ES cells contained alkaline phosphatase activity (Figure 4A). Immunophenotyping of the ES cells was carried out using a series of antibodies which detect cell surface carbohydrates and associated proteins found on human EC cells. The ES cells reacted positively in indirect immunofluorescence assays with

antibodies against the SSEA-4 and TRA 1-60 carbohydrate epitopes, and the staining patterns were similar to those observed in human EC cells (Figure 4B, C). ES cells also reacted with monoclonal antibody GCTM-2, which detects an epitope on the protein core of a keratan sulphate/chondroitin sulphate pericellular matrix proteoglycan found in human EC cells (Figure 4D). Like human EC cells, human ES cells did not express SSEA-1, a marker for mouse ES cells. Both cell lines were karyotypically normal and both were derived from female blastocysts.

Oct-4 is a POU domain transcription factor whose expression is limited in the mouse to pluripotent cells, and recent results show directly that zygotic expression of Oct-4 is essential for establishment of the pluripotent stem cell population of the inner cell mass. Oct-4 is also expressed in human EC cells and its expression is down regulated when these cells differentiate. Using RT-PCR to carry out mRNA analysis on isolated colonies consisting mainly of stem cells, we showed that human ES cells also express Oct-4 (Figure 5, lanes 2-4). The PCR product was cloned and sequenced and shown to be identical to human Oct-4 (not shown).

Example 3 - Differentiation of human ES cells in vitro

Both cell lines underwent spontaneous differentiation under standard culture conditions, but the process of spontaneous differentiation could be accelerated by suboptimal culture conditions. As with human EC cells, cultivation to high density for extended periods (4-7 weeks) without replacement of a feeder layer promoted differentiation of human ES cells. In high density cultures, expression of the stem cell marker Oct-4 was either undetectable or strongly downregulated relative to the levels of the housekeeping gene beta actin (Figure 5, lanes 5-7). Alphafetoprotein and human chorionic gonadotrophin were readily detected by immunoassay in the supernatants of cultures grown to high density. Alphafetoprotein is a characteristic product of endoderm cells and may reflect either extraembryonic or embryonic endodermal differentiation; the levels observed (1210-5806 ng/ml) are indicative of extensive endoderm present. Human chorionic gonadotrophin

secretion is characteristic of trophoblastic differentiation; the levels observed (6.4-54.6 IU/Litre) are consistent with a modest amount of differentiation along this lineage.

After prolonged cultivation at high density, multicellular aggregates or vesicular structures formed above the plane of the monolayer, and among these structures clusters of cells or single cells with elongated processes which extended out from their cell bodies, forming networks as they contacted other cells (Figure 3F) were observed. The cells and the processes stained positively with antibodies against neurofilament proteins and the neural cell adhesion molecule (Figure 4E and F). Contracting muscle was seen infrequently in the cultures. While contracting muscle was a rare finding, bundles of cells which were stained positively with antibodies directed against muscle specific forms of actin, and less commonly cells containing desmin intermediate filaments (Figure 6G and H) were often observed. In these high density cultures, there was no consistent pattern of structural organisation suggestive of the formation of embryoid bodies similar to those formed in mouse ES cell aggregates or arising sporadically in marmoset ES cell cultures.

Example 4 - Differentiation of human ES cells in xenografts

When HES-1 or HES-2 colonies of either early passage level (6; HES 1 and 2) or late passage level (HES-1, 14 and 27) were inoculated beneath the testis capsule of SCID mice, testicular lesions developed and were palpable from about 5 weeks after inoculation. All mice developed tumours, and in most cases both testis were affected. Upon autopsy lesions consisting of cystic masses filled with pale fluid and areas of solid tissue were observed. There was no gross evidence of metastatic spread to other sites within the peritoneal cavity. Histological examination revealed that the lesion had displaced the normal testis and contained solid areas of teratoma. Embryonal carcinoma was not observed in any lesion. All teratomas contained tissue representative of all three germ layers. Differentiated tissues seen included cartilage, squamous epithelium, primitive neuroectoderm, ganglionic structures, muscle, bone, and

glandular epithelium (Figure 6). Embryoid bodies were not observed in the xenografts.

Finally it is to be understood that various other modifications and/or alterations may be made without departing from the spirit of the present invention as outlined herein.

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FIGURE 1

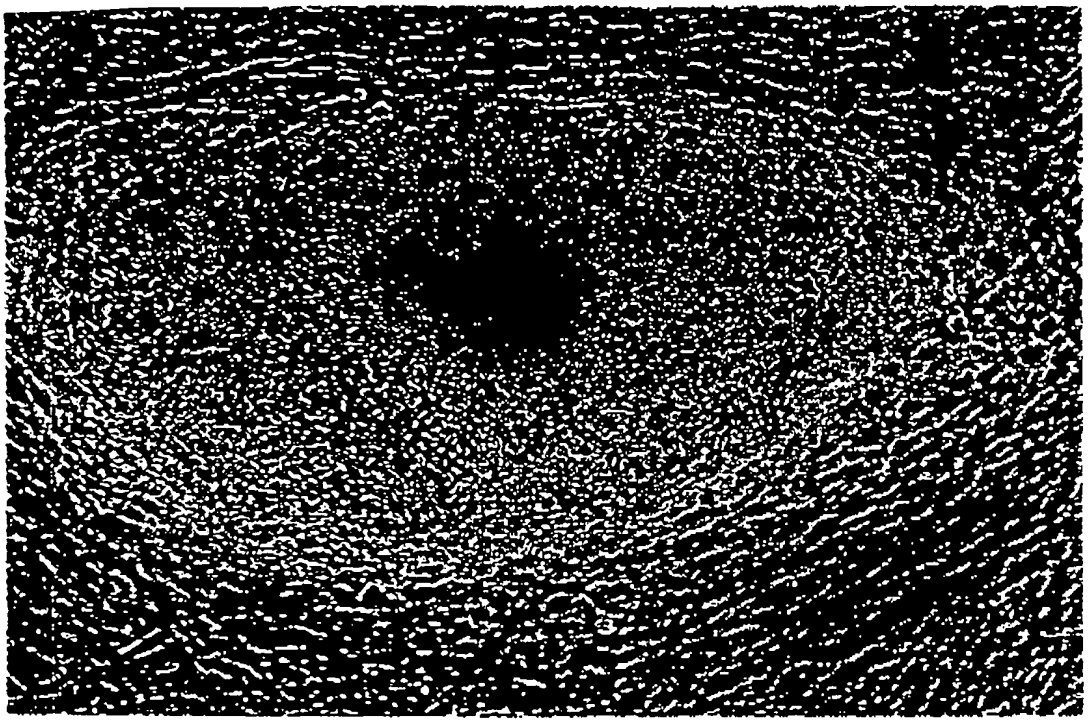

FIGURE 2

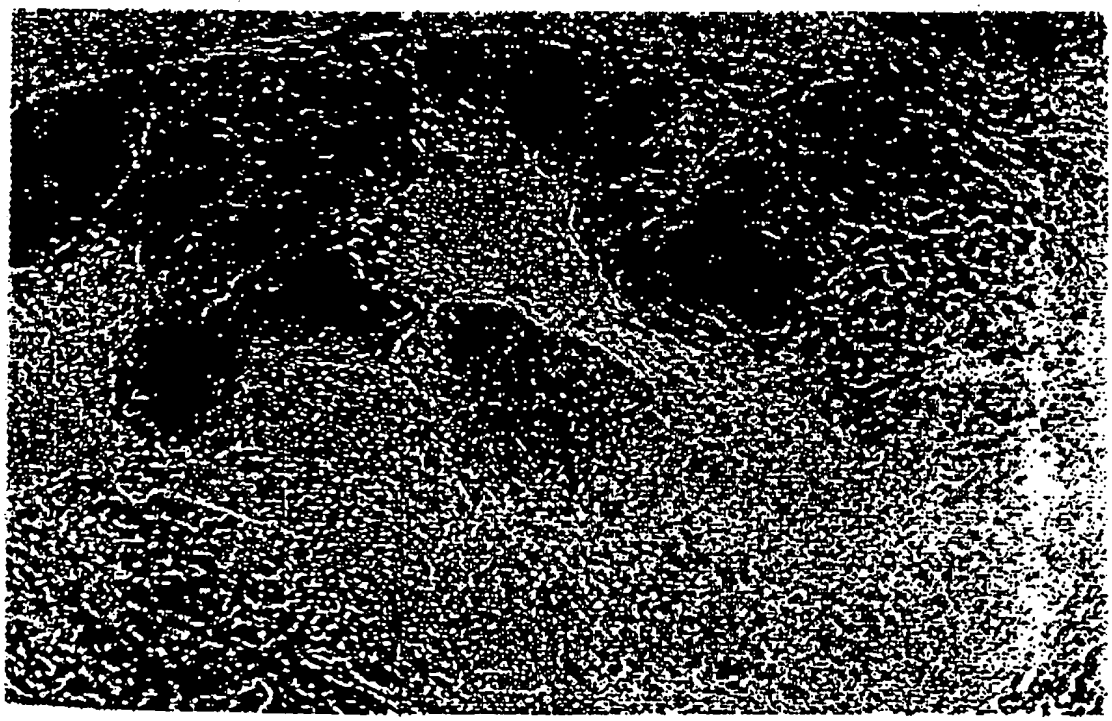

FIGURE 3

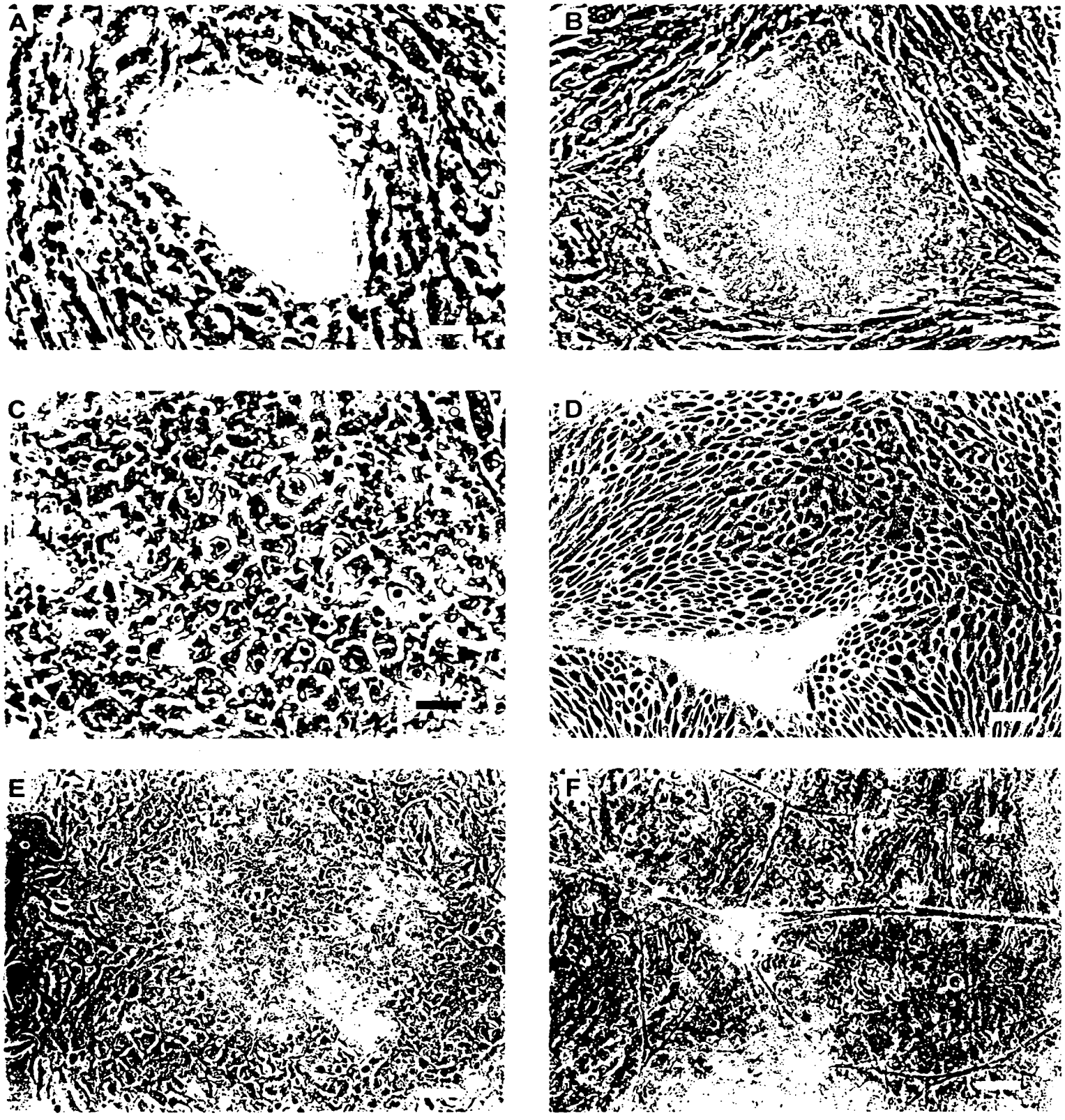

FIGURE 4

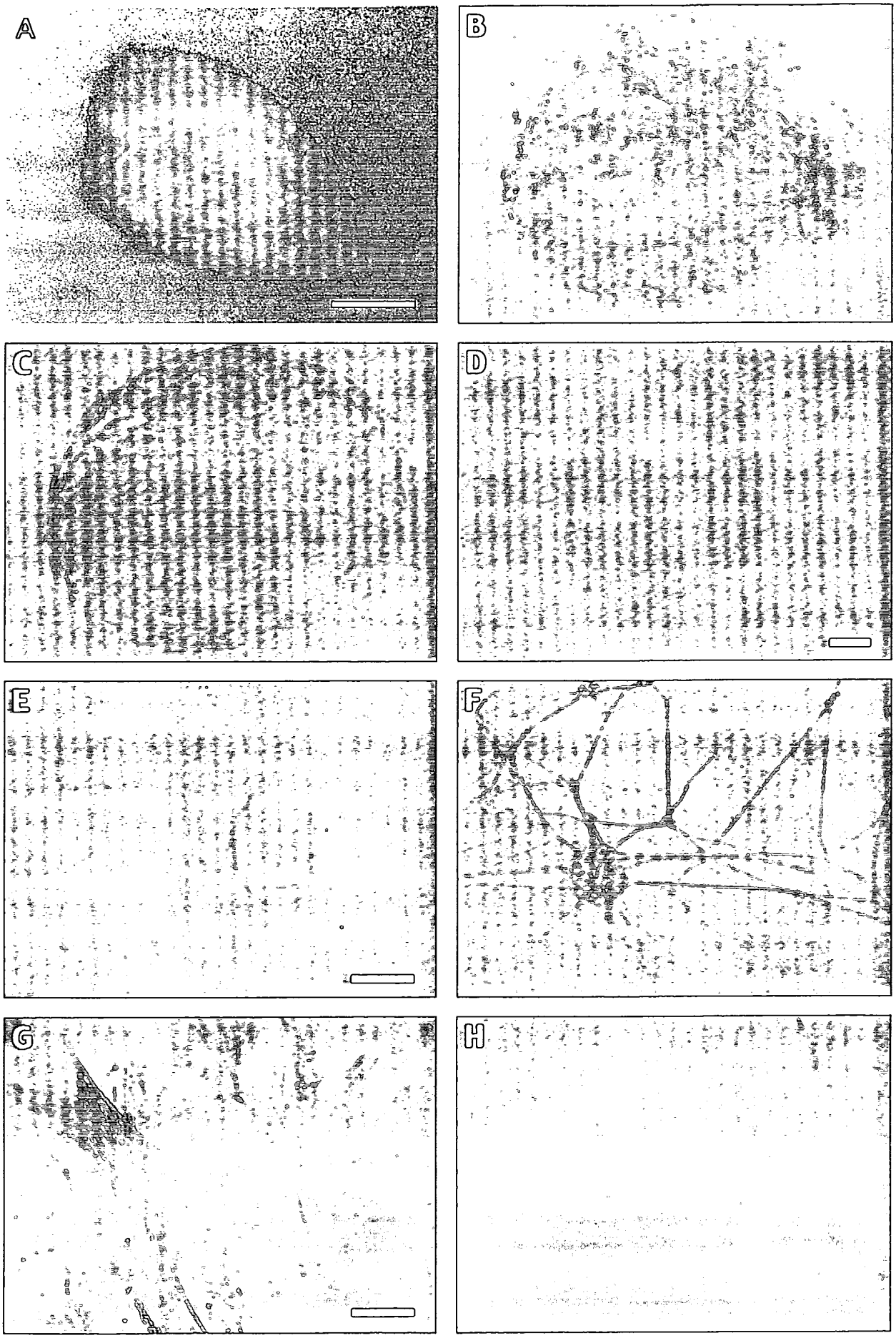

FIGURE 5

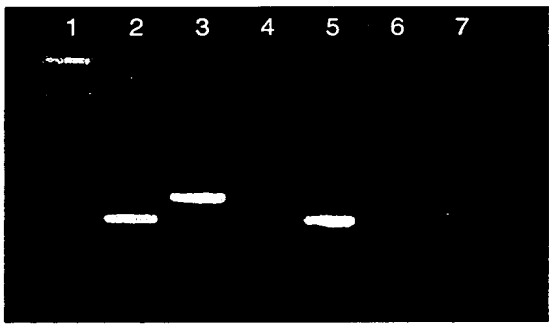

FIGURE 6